Aflatoxin M_1 in Milk: Evaluation of Methods

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Six published methods for the determination of aflatoxin M_1 in liquid and powdered milk were compared because a quantitative assay sensitive to 0.1 μg/L was needed for routine analysis. Each method was tested with both spiked and naturally contaminated samples at levels of 0.1, 0.5, and 1 μg/L or 1, 5, and 10 μg/kg; recoveries of M_1 were determined. Data revealed that 2 methods, one for liquid milk and one for powdered milk, had the desired sensitivity and recoveries. In the liquid milk method, M_1 is extracted and milk protein is precipitated simultaneously with methanol-water (4+1); in the powdered milk method, M_1 is extracted with acetone-water (70+30) and milk protein is then precipitated with a lead acetate solution. Both methods remove fats from the aqueous acetone or methanol solutions with hexane before partition of M_1 into chloroform. Aflatoxin M_1 is determined by thin layer chromatography of the chloroform extracts and either visual or densitometric measurement of mycotoxin on thin layer plates. Modifications were made to simplify the methods and to reduce the time required to complete the assays.

Aflatoxin M_1 was first detected in milk of cattle ingesting aflatoxin B_1-contaminated feed (1). In 1968, Purchase and Vorster (2) reported finding M_1 in 5 of 21 commercial milk samples in South Africa. A domestic survey of more than 400 milk samples by Brewington *et al.* (3) did not reveal a single instance of M_1 contamination; the method used by these workers was sensitive to 1 μg aflatoxin M_1/L. Since then, Kiermeier (4) found aflatoxin M_1 in raw milk from German farms and in commercial milk supplies at levels of less than 1 μg/L. When present in milk, this mycotoxin presents a potential hazard to human health and one that could be especially significant because milk is a major constituent of infant diets. Younger experimental animals are more affected by aflatoxin than are older animals (5). Without question, sensitive, reliable methods for detecting M_1 are needed. Methods for determining aflatoxin M_1 in milk should be capable of detecting levels as low as 0.1 μg/L (liquid milk) or 1 μg/kg (powdered milk). These sensitivity limits are essentially equivalent because 10 L liquid milk yields about 1 kg milk powder.

Several analytical methods have been published for determining aflatoxin M_1 in milk (6–10), but most were developed for animal feeding studies that resulted in milk samples with high M_1 levels and have not been tested with samples containing low M_1 levels. We compared 6 published methods in terms of recoveries of M_1 and detection of M_1 at levels of 0.1 μg/L or 1 μg/kg. Modifications that simplified procedures and reduced analysis time are described for the 2 best methods—one for liquid milk and one for powdered milk.

Experimental

Methods

The 6 methods compared are:

Method I: Masri *et al.* (6)

Method II: Purchase and Steyn (7), acetone-water (70+30)

Method III: Purchase and Steyn (7), acetone-chloroform-water (38+58+4)

Method IV: Jacobson *et al.* (8)

Method V: Roberts and Allcroft (9)

Method VI: Fehr *et al.* (10)

Sample Preparation and Assay

A naturally contaminated milk (NCM) powder (donated by Dr. I. F. H. Purchase when he was associated with National Institute for Nutritional Diseases, Pretoria, South Africa) was assayed in triplicate (1 g each) by Method II (7). The average aflatoxin M_1 concentration (215 μg/kg) that we determined compared favorably with the value supplied with this powder. NCM samples were prepared by mixing required quantities of stock NCM powder with uncontaminated freeze-dried milk powder or by blending stock powder with fresh whole milk (3 min) before extraction. Values for all M_1 recoveries from

NCM samples were determined by comparing M_1 concentrations in the NCM samples with theoretical M_1 concentrations that assumed 215 μg/kg for the stock NCM powder. Spiked milk samples were prepared by adding known volumes of standard aflatoxin M_1 solution (10 μg/ml, in acetonitrile) to either fresh whole milk or uncontaminated milk powder. Samples for all 6 methods were prepared identically so that results could be compared in an unbiased manner.

Duplicate spiked and NCM samples with 0.1, 0.5, and 1 μg M_1/L or 1, 5, and 10 μg/kg were assayed by each liquid or powder method. All analyses were done under subdued light to prevent degradation of aflatoxin M_1. Experimental procedures were as described in each liquid or powder method, except for thin layer chromatography (TLC) of chloroform extracts.

Thin Layer Chromatography

Dried residues from chloroform extracts produced by all 6 methods were dissolved in 100 μl chloroform in Teflon-lined, screw-capped vials (1 dram). Extracts were applied by syringe to TLC plates (Adsorbosil-1, 0.5 mm wet thickness, Applied Science Laboratories, Inc., State College, Pa.). Plates were developed in isopropanol-acetone-chloroform (5+10+85) (11), and aflatoxin M_1 was determined with a spectrophotodensitometer (SD-3000, Schoeffel Instrument Corp., Westwood, N.J.) under longwave UV light (365 nm) (11). Residues extracted by Method V (9) were dissolved in 500 μl chloroform, instead of 100 μl for TLC and densitometry.

Partition Study

Aqueous acetone (80+20) (100 ml) and aqueous methanol (1+1) (625 ml), equivalent to deproteinized extraction mixtures described in respective Methods II (7) and IV (8), were spiked at a level of 500 μg/L with standard M_1 solution. Each aqueous mixture was extracted with chloroform, as described in the 2 methods, and after each extract was evaporated to dryness, aflatoxin M_1 in the individual residues was determined by TLC and densitometry.

Recommended Methods

Fluid Milk

26.C09 ***Reagents and Apparatus***

See 26.001(k) and 26.031(b), plus the following:

(a) *Aflatoxin std solns.*—Approx. 1.0 μg aflatoxin M_1/ml $CHCl_3$. *See* 26.004–26.012. Use ϵ = 19,950 at max. 357 nm for aflatoxin M_1.

(b) *Vials.*—1 dram, 26.001(m).

26.C10 ***Extraction***

(*Caution: See* 46.005, 46.011, 46.056, and 46.066.)

Blend 75 ml fluid milk 3 min with 300 ml MeOH; add 25 g Celite, 26.002(c)(*1*), and blend addnl 30 sec. Filter by vac. thru 1 cm Celite layer on coarse fritted glass buchner funnel. Press bed of Celite and pptd casein, and wash with 75 ml MeOH.

Transfer filtrate and washings (ca 375 ml) to 1 L separator and add ca 225 ml 4% NaCl soln. Ext with three 100 ml portions hexane to remove fat and discard hexane. Ext with three 100 ml portions $CHCl_3$. (Centrf. 10–15 min at ca 3000 rpm to break emulsions.) Combine $CHCl_3$ exts, wash with 300 ml 4% NaCl soln, dry ext over anhyd. Na_2SO_4, transfer to 1 L r-b flask, and evap. to near dryness. Quant. transfer ext to 1 dram vial with $CHCl_3$. Evap. just to dryness under stream of N.

26.C11 ***Preliminary Thin Layer Chromatography***

Proceed as in 26.020(a), except weigh 50 g silica gel, coat plates with 0.5 mm thickness, and dry coated plates 1–2 hr at 105°; (b), except add 100 μl $CHCl_3$ to vial, spot 2.5, 5, and two 10 μl spots on imaginary line 2 cm from bottom edge of TLC plate, spot 2 μl std on top of one 10 μl sample origin spot as internal std, and place 100 ml isopropanol-acetone-$CHCl_3$ (5+10+85) in unlined developing tank; (c), except redissolve in calcd vol. $CHCl_3$; and (d), where W = ml sample extd and calcd concn is in μg/L.

26.C12 ***Quantitative Thin Layer Chromatography***

Proceed as in 26.038, except in (c) spot aflatoxin std to provide 3 ng M_1/spot. W = ml sample represented by final ext and concn is in μg/L.

Powdered Milk

26.C13 ***Reagent***

Lead acetate soln.—Dissolve 200 g $Pb(OAc)_2.3H_2O$ in H_2O with warming, add 2 ml HOAc, and dil. to 1 L with H_2O.

26.C14 ***Extraction***

(*Caution: See* 46.011, 46,046, 46.056, and 46.061.)

Quant. transfer 25 g milk powder to 500 ml g-s erlenmeyer contg 200 ml acetone-H_2O (7+3) and ca 150 two mm diam. glass beads. Shake flask 30 min on wrist-action shaker, 26.001(n). Filter suspension under vac. as in 26.C11, and rinse filter bed with 50 ml acetone-H_2O (7+3). Transfer quant. to 500 ml r-b flask and evap. solv. to ca 25 ml on rotary evaporator. Add 10 ml $Pb(OAc)_2$ soln, swirl to mix, and add 10 ml satd Na_2SO_4 soln. Transfer to 250 ml centrf. bottle, centrf. 20 min at 2000 rpm, and decant supernate into 250 ml separator. Resuspend ppt in 20 ml acetone-H_2O (1+4), repeat centrfg, and transfer.

Ext combined supernates with two 50 ml portions hexane, draining lower layer into 125 ml separator.

Table 1. Summary of methods

Method (ref.)	Extn app.	Extn solv.	Pptg agent	Column chromatgy
I (6)	Blender	MeOH-water (1+1)[a]		Yes
II (7)	Wrist shaker	Acetone-water (70+30)	Lead acetate	No
III (7)	Soxhlet	Acetone-$CHCl_3$-water (38+58+4)	None	No
IV (8)	Blender	MeOH-water (4+1)[a]		Yes
V (9)	Water bath	Acetone-water (2+1)	Heat	No
VI (10)	Soxhlet	Methanol	HCl	No

[a] Aflatoxin M_1 is extracted and milk protein is precipitated simultaneously with aqueous methanol extraction mixtures.

Ext with three 50 ml portions $CHCl_3$. (Centrf. 10–15 min at ca 3000 rpm to break emulsions.) Dry combined $CHCl_3$ exts over anhyd. granular Na_2SO_4, evap. to near dryness, and transfer to 1 dram vial. Evap. to dryness under stream of N.

Proceed as in **26.C11** and **26.C12**, except W = g sample in final ext and calcd concn is in μg/kg.

Results and Discussion

The 6 methods compared are summarized in Table 1. Methods I–III were designed to assay powdered milk, whereas Methods IV–VI were developed for liquid milk analysis. Powdered milk can be reconstituted with water and assayed by liquid milk methods, but the methods are more cumbersome to use because they require larger solvent volumes and, consequently, glassware of greater capacity. The basic steps are similar in 5 of the methods. Milk is extracted with an aqueous organic solvent mixture, and milk protein is precipitated. Clarified extracts are defatted with hexane and M_1 is partitioned into chloroform. Some procedures include column chromatography to purify extracts further before TLC analysis. Method VI (10) differs from the other 5 because liquid milk is treated with hydrochloric acid to separate aflatoxin M_1 and the curd from the whey fraction. The curd is refluxed in a Soxhlet apparatus with hexane to remove fats and with methanol to extract aflatoxin M_1. Since no organic solvent is used to extract the initial milk sample, many impurities present in whey are eliminated from TLC extracts.

Aflatoxin M_1 recoveries determined for spiked and NCM samples by all 6 methods are compared in Table 2. Aflatoxin M_1 could not be detected at the lowest level (1 μg/kg) with Method I (6) because fluorescent impurities that obscured M_1 zones were present in chloroform extracts developed on TLC plates. These contaminants contributed significantly to low M_1 recoveries and lack of sensitivity. Aflatoxin M_1 could not be detected at 0.1 μg/L by Method V (9). Evaporation of chloroform extracts resulted in oily residues that had to be dissolved in 500 μl chloroform to reduce viscosity for accurate spotting on TLC plates. The 5-fold dilution reduced the sensitivity of this method.

Aflatoxin M_1 recoveries decreased as mycotoxin concentration increased when samples were assayed by Method III (7). During the Soxhlet extraction, solvent channeled through the milk powder instead of completely saturating it. This condition may have been caused by the physical form of milk powder used to prepare the samples. Some freeze-drying processes produce hard, crystalline powders, whereas others give soft, electrostatic powders. Our equipment produced the soft powders.

Method II (7) was best for assaying powdered milk samples. Aflatoxin M_1 was easily detected at 1 μg/kg and was satisfactorily recovered from all NCM samples (>90%). Less M_1 was recovered from spiked samples (65–75%); however, M_1 was recovered quantitatively when added directly to the extraction solvent instead of to the milk powder. The difference in recoveries from powder and solvent suggests that M_1 binds to milk protein. Similar losses occurred when powder was spiked with standard solutions prepared with chloroform or acetonitrile.

The best method to determine M_1 in liquid milk is Method IV (8). We recovered from 73 to 79% (0.1 μg/L) and 90% or more (0.5 and 1 μg/L) with both spiked and NCM samples. In contrast to low recoveries from powdered milk, aflatoxin M_1 is readily recovered from liquid milk that has been spiked with standard solutions. Analytical data for Method IV were acquired by omitting the column chromatographic step for several reasons: Calcium chloride-Celite columns were difficult to

Table 2. Comparison of aflatoxin M_1 recoveries from spiked and naturally contaminated samples by liquid and powdered milk methods[a]

	M_1 recovered, %							M_1 recovered, %			
	Powdered milk methods							Liquid milk methods			
	I		II		III			IV		V	
$\mu g\ M_1/kg$[b]	SM	NCM	SM	NCM	SM	NCM	$\mu g\ M_1/L$[b]	SM	NCM	SM	NCM
10	20	57	65	94	68	53	1	99	105	86	91
5	30	49	67	95	102	78	0.5	89	98	40	75
1	ND	ND	74	90	102	92	0.1	73	79	ND	ND

[a] Determined by TLC of chloroform extracts and densitometry of developed plates. Each value represents an average of 2 determinations. Aflatoxin M_1 recoveries for NCM samples assume that the concentrated NCM powder used to prepare the samples contained 215 μg M_1/kg. Methods and references given in Table 1. Abbreviations: SM = spiked milk; NCM = naturally contaminated milk; ND = not detected.

[b] Liquid milk contains 10% solids; therefore, equivalent sample concentrations are represented by $\mu g/kg = 10\ \mu g/L$.

prepare, and aflatoxin M_1 was neither completely eluted from the column (ca 50%) nor regularly eluted in a specified fraction. Since the TLC solvent system, isopropanol-acetone-chloroform (5+10+85) (11), clearly separated contaminants from M_1 zones on plates, a column cleanup step was not necessary. A considerable reduction in analysis time is saved by omitting this step.

Data for samples analyzed by Method VI (10) are not included in Table 2 because the lowest level at which M_1 could be detected was 1 $\mu g/L$. While only 25% of the total M_1 in the milk was recovered from the curd fraction, an additional 25% was discovered in the whey fraction. Other extracting solvents (methanol-water, acetone-water, and acetone), precipitating agents (ammonium sulfate and ethanol), and extracting apparatus (blender) failed to remove more than 55% of the total M_1 in liquid milk. As postulated with spiked milk powders, M_1 may be binding to the milk protein, or it may be occluding in the curd protein during the precipitation step.

Additional investigations with Methods II and IV were made to simplify procedures and reduce analysis time. Partition studies disclosed that one less chloroform extraction was needed than specified (Table 3). Almost all aflatoxin M_1 ($\geqq 97\%$) can be extracted with 3 partitions from aqueous methanol (Method IV (8)) or 2 partitions from aqueous acetone (Method II (7)) with chloroform, even when aqueous mixtures contain levels of 500 $\mu g/L$. Aqueous methanol extracts were satisfactorily defatted in our laboratory with 3 hexane extractions. Since the ratio of volumes of aqueous methanol-hexane is large (6+1) and since fat content in milk varies with different locations,

Table 3. Partition of aflatoxin M_1 into chloroform from aqueous extraction solvents

	M_1 recovered, %[a]	
Chloroform partition	Acetone-water (20+80)[b]	Methanol-water (1+1)[c]
1	96	67
2	2	23
3	0.1	7
Total	98	97

[a] Determined by TLC of chloroform extracts and densitometry of developed plates.

[b] Solvent mixture (50 ml) for Method II (7) was spiked with aflatoxin M_1 (500 $\mu g/L$) and extracted with 50 ml volumes of chloroform.

[c] Solvent mixture (625 ml) for Method IV (8) was spiked with aflatoxin M_1 (500 $\mu g/L$) and extracted with 100 ml volumes of chloroform.

defatting sample extracts by Method IV (8) may require changes in volumes. Larger volumes of hexane (150–200 ml) should be used if an oily residue remains after evaporation of chloroform. Aqueous acetone extracts (Method II (7)) are sufficiently defatted with 2 hexane partitions because equal volumes of hexane and aqueous extract are used. When emulsions were formed during either hexane or chloroform partitions, centrifugation (15 min at 2000 rpm) always separated the layers.

Several different brands of fresh whole milk and all available NCM samples were extracted by Methods II and IV to reveal any ingredient that might interfere with assays. Although fluorescent zones were detected on TLC plates, none

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was present in the area of aflatoxin M_1 zones. No aflatoxin M_1 was found in any commercial sample. Milk samples containing high levels of M_1 (200 μg/kg) were prepared and quantitatively analyzed by both methods without difficulty.

The 2 modified methods (as described in *Experimental*) were recommended for testing in an international collaborative study conducted by the International Union of Pure and Applied Chemistry (IUPAC) under the direction of Dr. I. F. H. Purchase (Purchase and Stubblefield, manuscript in preparation).[1]

Note: During the preparation of this manuscript, J. D. McKinney (*J. Amer. Oil Chem. Soc.* **49**, 444–445 (1972)) published a modified Jacobson method (Method IV) for determining aflatoxin M_1 in milk that is similar to the one we describe here.

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[1] On the basis of the collaborative results in this report, the methods for the determination of aflatoxin M_1 in fluid and powdered milks were adopted as official first action; *see* (1973) *JAOAC* **56**, 400.

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